



Enhanced Growth in the Clonal Propagated Plants

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Abstract:

Micro propagation has the potential to provide very high multiplication rates of selected true genotypes. It allows rapid production of high quality, disease free and uniform planting materials irrespective of weather or season. The paper highlights studies on the same, outlines potential of using single to multiple co-cultures combinations of microbe that were in symbiotic relationship with plants and thus promoting greater nutritional assimilation by plants. The microorganisms were obtained from six different soil locations and were identified using gram staining and IMVIC techniques. Later they were enumerated using the serial dilutions. These bacteria were then inoculated to the tissue cultured plants in a series of triple combination and in different concentrations. The result obtained was compared against the control. The key parameters of plant growth are reported and discussed. There is enough experimental evidence with bacteria (bacterization) and vesicular arbuscular mycorrhiza (mycorrhization) inoculations to recommend utilization of this technology in commercial micropropagation. There was an increased growth in all the macro and micro nutrients and protein formation and increased the immunity and performance of the cultured plants, fidelity test was conducted to prove that plants had the same genetic make up. Techniques have found increasing use in the conservation of agricultural crops and threatened plants in recent years and this trend is likely to continue as it is significantly contributing to agricultural growth.

Keywords: *Micropropagation, microbe combinations, inoculation.*

Introduction:

The vegetative micropropagation of fruit, ornamental and forest species is the main tool for producing genetically homogenous plants in good phytosanitary conditions and in large numbers^[1] Micropropagation protocols have advantages over traditional clonal propagation methods including high multiplication rates and production of disease free plants^[2] Shoot tip culture was considered a better method for micropropagation, since it produced plants phenotypically similar to the mother plant and gave a

much more rapid multiplication rate when compared to the other procedure^[3] In the last 10 years, micropropagation has shown spectacular development. However, at present the widespread use of micropropagation is restricted for several reasons, one of these being that a high percentage of micropropagated plants can be lost or damaged in the acclimatization stage. Due to the special environment *in vitro*, it is difficult to produce plants which can be acclimatized to the outside environment^[4] It is impossible to discuss the acclimatization of tissue cultured plants without evaluation of the intrinsic qualities of the micropropagated material. Compared to traditionally propagated plants, tissue cultured plants are aberrant from the anatomical and physiological point of view. Different approaches allow to correct this abnormal behavior, of "normal looking" plants while they are still in culture. Most corrections occur with respect to water, housekeeping and photosynthetic behaviour of micropropagated material. Rooting can be induced *in vitro*. Different greenhouse or growing room setups have been evaluated under experimental and commercial conditions^[5] Leafy or chlorophyllous explants of a number of plant species currently micropropagated have been found to have high photosynthetic ability^[6] During the first days after transplanting micropropagated plants to greenhouse conditions, *in vitro* leaves are the only source to cover metabolic demands and to sustain plants' adaptation and regrowth^[7] The greenhouse and field have substantially lower relative humidity, higher light levels, and septic environments that are stressful to micropropagated plants compared to *in vitro* conditions. Most species grown *in vitro* require an acclimatization process in order to insure that sufficient numbers of plants survive and grow vigorously when transferred to soil^[8]. After transfer from the *in vitro* to the *ex-vitro* conditions the plantlets have to correct the above-mentioned abnormalities. Therefore, after *ex-vitro* transplantation plantlets usually need some weeks of acclimatization^[9]. During acclimatization, the rooted plantlets develop fully functional roots and leaves, and shoot growth is initiated or accelerated. Growth of micropropagated plants is vigorous and seems

invariably to be characterized by greater branching. These characteristics affect the field performance of micropropagated plants, a subject that is being studied extensively in fruit crops and forest trees ^[10].

In this review, six types of bacteria's were collect from different regions of Andhra Pradesh, identified, isolated, purified and were then inoculated to various cultured plants in combination of three sets. Different bacterial combinations in different concentrations showed difference in the growth of the root length, shoot length and number of leaves. These changes were observed, compared and recorded.

Materials and methods:

Isolation and screening and of microorganisms:

Serial dilution method was used to isolate bacteria from soil samples collected from six districts of Andhra Pradesh. When these serial dilutions are plated, discrete colonies will appear. Later the gram staining and IMVIC tests were performed and the results were noted.

DNA Extraction & Enumeration of Bacteria:

DNA Extracted from bacteria was serially diluted by a factor of 3 and amplified in triplicate according to the improved PCR protocol. The Number of amplifiable target DNA sequences corresponding to the number of bacterial cells was determined according to the most-probable-number (MPN) technique ^[11]

Inoculation: The six bacteria that were isolated from the soil samples were inoculated to the micropropagated plants. Inoculum densities were optically adjusted at 600 nm and verified by standard plate counts. The Cells were rinsed with sterile distilled water and suspended in less than 100µl of water to avoid dilution of the soil sample ^[11]

At the end of all these tests PGRP was given to each of the micropropagated plants in three bacterial combinations from which 20 combinations were obtained, i.e T0- The control plant that was left untreated. This is required to compare plants with bacterial action and helping in a better understanding of the effects of the biological agents.

- T1- Rhizobium + Acetobacter + Azospirillum (2.41x10⁵ CFU/ml, 2.0x10⁷CFU/ml , 1.82x10⁵CFU/ml)
- T2- Rhizobium + Acetobacter + Pseudomonas (2.41x10⁵ CFU/ml, 2.0x10⁷CFU/ml,1.42x10³cfu/ml)
- T3- Rhizobium + Acetobacter + PSB (2.41x10⁵ CFU/ml, 2.0x10⁷CFU/ml, 1.5x10⁶CFU/ml)
- T4- Rhizobium + Acetobacter + VAM (2.41x10⁵ CFU/ml, 2.0x10⁷CFU/ml, 10gm/plant 50 spores per gram)
- T5- Acetobacter + Azospirillum + Pseudomonas (2.0x10⁷CFU/ml , 1.82x10⁵CFU/ml, 1.42x10³cfu/ml)
- T6- Acetobacter + Azospirillum + PSB (2.0x10⁷CFU/ml , 1.82x10⁵CFU/ml, 1.5x10⁶CFU/ml)
- T7- Acetobacter + Azospirillum + VAM (2.0x10⁷CFU/ml , 1.82x10⁵CFU/ml, 10gm/plant 50 spores per gram)
- T8- Azospirillum + Pseudomonas + PSB (1.82x10⁵CFU/ml, 1.42x10³cfu/ml, 1.5x10⁶CFU/ml)
- T9- Azospirillum + Pseudomonas + VAM (1.82x10⁵CFU/ml, 1.42x10³cfu/ml, 10gm/plant 50 spores per gram)
- T10- Pseudomonas + PSB + VAM (1.42x10³cfu/ml, 1.5x10⁶CFU/ml, 10gm/plant 50 spores per gram)
- T11- Rhizobium + Azospirillum + Pseudomonas (2.41x10⁵ CFU/ml, 1.82x10⁵CFU/ml, 1.42x10³cfu/ml)
- T12- Rhizobium + Azospirillum + PSB (2.41x10⁵ CFU/ml, 1.82x10⁵CFU/ml, 1.5x10⁶CFU/ml)
- T13- Rhizobium + Azospirillum + VAM (2.41x10⁵ CFU/ml, 1.82x10⁵CFU/ml, 10gm/plant 50 spores per gram)
- T14- Rhizobium + Pseudomonas + PSB (2.41x10⁵ CFU/ml, 1.42x10³cfu/ml, 1.5x10⁶CFU/ml)
- T15- Rhizobium + Pseudomonas + VAM (2.41x10⁵ CFU/ml, 1.42x10³cfu/ml, 10gm/plant 50 spores per gram)
- T16- Acetobacter + Pseudomonas + PSB (2.0x10⁷CFU/ml,1.42x10³cfu/ml, 1.5x10⁶CFU/ml)
- T17- Acetobacter +Pseudomonas + VAM (2.0x10⁷CFU/ml,1.42x10³cfu/ml, 10gm/plant 50 spores per gram)
- T18- Azospirillum + PSB + VAM (1.82x10⁵CFU/ml, 1.5x10⁶CFU/ml, 10gm/plant 50 spores per gram)
- T19- Rhizobium + PSB + VAM (2.41x10⁵ CFU/ml, 1.5x10⁶CFU/ml, 10gm/plant 50 spores per gram)
- T20- Acetobacter + PSB + VAM (2.0x10⁷CFU/ml, 10gm/plant 50 spores per gram)

These combinations were inoculated to the roots of the micropropagated plants and the results were compared against control.

All the above 20 combinations were inoculated to the roots of the micropropagated plants in various concentrations of 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, for each combination and parameter 10 plants were taken and the mean of the values of the root length, shoot

length, leaf primordia and no of leaves were recorded every week and it was continued for 12 weeks the results were compared against the control. The inoculums of 2.5% concentration had showed

maximum growth thus considering it as an optimum concentration.

Nutrient analysis: The micro and macro nutrient analysis was done by Perkin- Elmer Analyst 300 single beam atomic spectrometry, the plants that were treated with PGPR showed enhanced nutrient content in it and documented^[12].

Genetic stability test: As there were increased levels of nutrients, proteins and growth of the micropropagated plants with the inoculation of PGPR, Fedility test was done to confirm the genetic stability of these plants and it is documented^[13]

Enumeration of Bacteria: Each bacterium showed varied colony forming units, the actual number of CFU was analyzed for the inoculation considering the volume of loop to be 0.01ml. The viable counts ranged in the soil samples were Rhizobium (2.41×10^4 CFU/ml), pseudomonas (1.42×10^3 CFU/ml), PSB(1.5×10^6 CFU/ml), Azospirillum(1.82×10^5 CFU/ml), Acetobacter(2.0×10^7 CFU/ml), VAM (10gm/plant 50 spores per gram)

Survival rate:

The micro propagated plants, showed a good growth in all the concentrations especially in 2.5%, but when the concentrations of inoculums (micro-organisms) increased from 3% and beyond, the plantlets showed high mortality rate.

Results and discussion:

It was seen that the combination in T2 showed a higher range of root length and T15 showed a higher growth range of shoot length. The presence of rhizobium pseudomonas, acetobacter and VAM in the given combinations has showed a higher growth range. The concentration of 2.5% showed maximum output in all the combinations. Hence it is proved that the cake of these useful bacteria combined together gives an excellent growth pattern and shows the relationship between the soil and the plants.

TABLE 1: Effect of Inoculum on Root Length:

Plantlets	0.5%	1.0%	1.5%	2.0%	2.5%
T0	5.0	5.0	5.0	5.0	5.0
T1	9.0	8.2	2.5	5.4	12.6
T2	9.0	8.8	2.8	5.9	13.9
T3	9.4	8.5	2.9	5.8	13.4
T4	8.2	7.9	3.0	6.0	12.2

T5	7.6	6.4	2.5	5.5	10.8
T6	7.0	6.0	2.5	5.5	10.0
T7	6.4	6.3	3.5	5.3	6.9
T8	6.6	6.4	3.3	4.3	7.7
T9	6.5	6.6	3.8	4.4	7.7
T10	8.8	7.9	7.7	6.4	13.6
T11	5.1	5.9	5.3	5.1	9.9
T12	4.0	4.9	5.3	5.2	9.7
T13	6.1	5.2	5.3	6.1	10.1
T14	4.5	5.5	7.0	6.3	7.8
T15	4.4	5.9	6.9	6.4	7.9
T16	7.3	6.2	2.5	5.0	10.7
T17	6.6	6.4	3.8	4.9	7.0
T18	7.6	7.5	6.4	5.3	10.1
T19	9.2	8.0	5.0	6.4	13.8
T20	8.5	7.5	2.5	6.5	12.5

This table shows that the 2.5% concentration showed the highest growth and in this the T2 combinations, that is Rhizobium + Acetobacter + Pseudomonas showed the highest root growth

Colonized plantlets had more extensive root systems and better shoot growth than control plants. The VAM symbiosis reduced the plantlet osmotic potential. This response may be a useful pre-adaptation for plantlets during transfer to the acclimatization stage^[14] The ability of Azospirillum to fix the atmospheric nitrogen in to the soil and make it available to the plants is an important trait in sustainable farming for increasing crop yield^[15]

Figure 1: Untreated control plant T0, growth



TABLE 2: Effect of Inoculum on Shoot Length:

Plantlets	0.5%	1%	1.5%	2%	2.5%
T0	9.2	9.2	9.2	9.2	9.2
T1	20.1	15.5	11.6	11.4	17.6
T2	18.2	15.0	10.6	10.4	19.0
T3	20.9	17.1	13.1	11.9	19.1
T4	17.7	16.2	12.9	11.3	18.8
T5	7.7	6.9	2.9	6.0	11.5
T6	7.2	6.9	2.4	6.3	11.2
T7	18.4	14.8	15.2	13.8	19.1
T8	13.0	18.3	14.1	13.0	19.1
T9	12.0	15.0	14.0	13.0	16.6
T10	12.0	16.4	14.9	13.3	16.8
T11	10.2	12.3	15.4	16.4	17.4
T12	9.2	16.3	15.3	16.1	17.4
T13	8.8	16.2	11.4	15.8	16.8
T14	9.4	9.9	13.6	12.8	15.5
T15	9.8	9.8	13.4	12.4	19.2
T16	7.4	7.0	2.6	5.5	11.0
T17	13.2	16.1	14.8	15.4	17.0
T18	12.0	16.6	14.3	13.4	16.2
T19	15.0	16.4	13.9	12.2	17.6
T20	18.2	16.4	16.6	11.8	18.4

This table shows us the result for the shoot growth in the appropriate bacterial combinations against the concentrations. The 2.5% showed the maximum of which the T15 combination, that is Rhizobium + Pseudomonas + VAM showed the highest growth.

Introduction of the mycorrhizal fungus at the time of transfer of plantlets from axenic conditions to ex vitro conditions improved shoot and root growth; enhanced the shoot: root ratio; increased the concentration and/or content of N, P, and K in plant tissues; and helped plants to tolerate environmental stress at transplanting. Inclusion of soil as a component of the potting medium appeared to favor mycorrhiza formation and effectiveness^[16].

TABLE 3: LEAF PRIMORDIAL

Plantlets	0.5%	1%	1.5%	2.0%	2.5%
T0	1	1	1	1	1
T1	0	1	1	0	1
T2	0	1	1	1	1
T3	0	1	1	0	1
T4	0	1	1	0	1
T5	2	1	0	1	2
T6	2	1	0	0	1
T7	2	2	1	2	1
T8	2	1	1	1	2
T9	2	2	1	1	2
T10	0	1	1	1	2
T11	1	2	2	1	1
T12	1	2	1	1	1
T13	0	2	2	1	2
T14	1	2	2	1	2
T15	1	1	2	1	2
T16	2	1	2	1	2
T17	2	0	1	0	1
T18	2	2	1	1	2
T19	0	1	1	2	2
T20	0	1	1	0	2

The 2.5% shows the highest leaf primordial. Therefore we can safely say that the three bacterial combinations helped to improve the growth of these micropropagated plants.

Figure 2: Highest growth shown in the 2.5% conc.



TABLE 4: NUMBER OF LEAVES:

Plantlets	0.5%	1.0%	1.5%	2.0%	2.5%
T0	5	5	5	5	5
T1	7	6	5	7	9
T2	8	6	5	7	8
T3	9	7	5	7	9
T4	7	5	5	7	9
T5	8	5	5	5	8
T6	7	5	5	6	7
T7	7	5	5	5	7
T8	6	4	5	5	6
T9	7	5	4	5	7
T10	4	3	2	3	4
T11	4	6	6	4	7
T12	3	9	7	5	6
T13	4	5	4	5	7
T14	6	6	6	5	6
T15	7	5	5	6	7
T16	6	4	4	4	7
T17	6	5	5	6	7
T18	6	4	4	6	6
T19	5	5	6	5	6
T20	8	5	5	8	8

This table gives us the maximum growth of the 2.5% concentration in the root length, shoot length, leaf primordial and number of leaves.

The 2.5% concentration showed the highest content. The increase in the number of leaves in turn increased the photosynthetic activity as stated earlier. The purpose of this work was to evaluate the role of microorganisms present in the soil in enhancing the mortality and growth rate of micropropagated plants; Various soil bacteria have been isolated using different methods like screening and staining from natural resources in various parts of Andhra Pradesh. The micropropagated roots of MUSA PARADISIACA were inoculated with single bacteria. Plants induced with the bacteria Rhizobium showed more root length, shoot length, leaf primordial and number of leaves. Advancing the experiment dual combinations of organisms was

used. There was enhanced growth in the root seen which was inoculated with the dual combination of Rhizobium and Pseudomonas, in case of shoot length it was Azotobacter & Pseudomonas, No of leaves it is Azotobacter & Pseudomonas. Progressing that experiment we have taken the same bacteria's in a combination of 3 and 4 sets. As seen from the results, the 2.5% showed the maximum growth in all the combinations. The maximum root length was shown by the T2 combination (Rhizobium + Acetobacter + Pseudomonas) owing to the nitrogen fixing quality of these bacteria's also the availability of growth promoting factors. The maximum shoot length was shown by T15 combination (Rhizobium + Pseudomonas + VAM) owing to plant-growth promoting characteristics and phosphor uptake qualities. The research on the same microorganisms in different combinations also showed the maximum growth in 2.5% concentration. Hence it was proved that plants improve the fertility status of the soil and contribute to plant growth. Those using tissue culture for multiplication or transformation are concerned to produce microplants that are 'fit for the purpose', that is, free of specified diseases, vigorous, developmentally normal and genetically true to type^[17]. The response is useful for pre-adaptation for in vitro developed plantlets during transfer to the acclimatization stage^[14]. This in view of complementary functions of acetobacter in nitrogen fixation, aspergillus in phosphate solubilization and VAM in multiple functions^[18] The potential of micropropagation for mass scale propagation has raised high hopes and a lot of research has been focused on the development of protocols for large and rapid scale propagation^[19]. A protocol for hardening of micropropagated plants of *Leucaena leucocephala* in vitro is described which ensures up to 85% survival of plants following their transfer to pots or garden beds^[20]

Conclusion:

This research concludes the beneficial effect of the selected microbial agents to increase the growth and survival rate of the micropropagated plants by inoculating them into it. Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings. Plants have extraordinarily high fecundity rate and also increases mortality rate. Therefore we can conclude that these bacterial combinations provide the best plant growth in terms of root, shoot, number of leaves etc.

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